Pharmacogn J. 2021; 13(2): 325-331
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
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Original Article

Production of Secondary Metabolites and its Antibacterial and Antioxidant Activity During the Growth Period of Endophytic Fungi Isolated from Gall Rust Sengon Plants

Alfi Rumidatul[1], Noor Rahmawati[1,*], Sopandi Sunarya[1]

ABSTRACT

Introduction: In our previous study, secondary metabolites of endophytic fungi isolated from gall rust sengon showed their bioactive activity against antibacterial and antioxidant, but only gave little effect to antifungal activity. Endophytic fungal culture extracted from gall rust of sengon (*Falcataria moluccana* Miq. Barneby and J. W. Grimes**)** has the ability on inhibiting *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Escherichia coli*. To increase the production of bioactive compounds, this research was focused on isolates which produced the highest activity compounds, and sought the optimal fermentation conditions in the production of bioactive compounds. **Methods:** The fermentation process was carried out on PDB liquid media for 21 days, under shaker conditions, at room temperature, and sampling measurements were hold every 3 days. Bioassay were carried out against *B. subtilis*, *P. aeruginosa,* and *E. coli*. **Results:** The early stages fungal growth was adaptation stage, continued by 1-6th days of exponential growth period. Day 6-12th was stationary growth and day 15-21th were cell death periode. The highest secondary metabolite production was achieved at stationary periodes, that was occured at days 9-15th, and high antibacterial activity was produced on days 9-12th. **Conclusion:** The best production secondary metabolite that has high activity was at day 9-12th.
Key words: Endophytic fungi, Secondary metabolite, Antibacteri, Antioxidant, Gall rust.

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History

- Submission Date: 31-10-2020;
- Review completed: 12-11-2020;
- Accepted Date: 04-12-2020.

DOI : 10.5530/pj.2021.13.42

Article Available online

http://www.phcogj.com/v13/i2

INTRODUCTION

Innovation of natural products for antibiotics and antioxidants is very urgent, because the emergence of antibiotic-resistant microorganisms that require inventive research and development strategies. Endophytic fungus isolated from the gall rust of *F. moluccana* have the ability to produce antimicrobial compounds and antioxidants. Thirty-four endophytic fungi were isolated from the gall rust of the *F. moluccana*, screened and evaluated for their ability to produce antimicrobial and antioxidants compounds. Antimicrobial activity was found in at least one or more pathogenic microbes (bacteria and fungi) tested. Endophytic fungal culture extract from the gall rust of the *F. moluccana* has the ability to inhibit *B.subtilis, P. aeruginos*a and *E. coli*.[1] To further increase the production of bioactive compounds, this research is focused on isolates which in the previous studies produced the highest compound and high activity, and sought optimal fermentation conditions in production.

Endophytic fungi are microorganisms living inside plants and are considered a promising source of novel and natural biologically active compounds.[2] Endophytic fungi could be found in every plant, because no studies had shown the presence of plant species without endophytes. High species diversity was another characteristic of endophytic microbes. The production of secondary metabolites in fungi can be affected by medium composition, pH, temperature, agitation, and lighting.[3] According to Deduke *et al.* [4] the factors affecting secondary metabolite production in fungi are geographical factors, dehydration, lighting, drought, growth medium, nutrient availability, pH, genes expression and carbon sources. Optimization of antioxidant compound production was carried out to obtain optimum condition for endophytic fungal growth which could produce secondary metabolite with bioactive compound having high antioxidant activity. Biosynthesis of secondary metabolites was directly related to culture conditions and length of incubation time, including biomass present in the production phase and duration of incubation.[5] Suitable growth conditions strongly supported the production of secondary metabolites in endophytic fungi. Secondary metabolites were a kind of compound that was produced or synthesized in cells at a certain growth or stress level. These compounds were produced in little amounts to defend themselves from their habitats threat, and did not play a major role in primary metabolic processes. To achieve this aim, optimum environmental conditions were required for the growth of endophytic fungi and the production of secondary metabolites, especially antioxidant compounds.

MATERIAL AND METHODS

Place and time

The study was conducted at the microbiology laboratory of the ITB Bioscience and Biotechnology Research Center, and was held from January - October 2019.

Cite this article: Rumidatul A, Rahmawati N, Sunarya S. Production of Secondary Metabolites and its Antibacterial and Antioxidant Activity During the Growth Period of Endophytic Fungi Isolated from Gall Rust Sengon Plants. Pharmacog J. 2021;13(2): 325-31.

Materials

The materials used in this study was a gall rust tumor from the sengon plant obtained from ITB Jatinangor campus. Other materials used were Nutrient Agar (NA), Nutrient Broth (NB), Potato Dextro Agar (PDA), Potato Dextrose Broth (PDB), gram-positive bacteria (*Bacillus subtilis* ATCC6633), gram-negative bacteria (*Pseudomonas aeruginosa* ATCC9027 and *Escherichia coli* ATCC8939). Organic solvent that were used in this research was ethyl acetate. DPPH was used as a source of radical for antioxidan activity test.

Isolation of endophytic fungi

Isolation of endophytic fungi was carried out as follows: endophytic fungi isolated from gall rust tumors from Sengon Plant. Gall was cut approximately 1 cm long and rinsed with distilled water for surface sterilization and then sequentially washed with 70% ethanol (1 minute), 5.25% sodium hypochlorite (15 minutes) and rinsed again with 70% ethanol for 1 minute followed by rinsing with sterille aquadestilata (three times). Gall was cut in the middle and then placed on PDA medium which had been added with antibiotics (200 µg of chloramphenicol) and incubated at 28 ° ± 1 ° C (room temperature) for about 1-3 weeks. The mycelium derived from the sample specimens was purified and cultured under the same conditions.[6]

Fermentation and extraction of endophytic fungi

The fungal endophytes were cultivated on (PDB) by placing 4 agar blocks of pure culture (10 mm in diameter) of actively growing culture in 250 ml erlenmeyer flasks containing 100ml of the medium. The flasks were incubated in shaker condition for 3 weeks at (21 days) 27±1°C. The culture was filtered through filter paper to remove the mycelial mats. The liquid broth was collected and extracted with equal volume of ethyl acetate l in a separating funnel by vigorous shaking for 1hour. The cell mass was separated and weight to obtain weight of micellium. The solvent was evaporated and the resultant compound was dried with MgSO4 and concentrated to yield the crude extracts. The crude extracts were then dissolved in methanol for antibacterial bioassay and antioxidant test.[6]

Antioxidants activity test (2,2-Diphenyl-1-pikrilhidrazil (DPPH) Scavenging Tests)

DPPH radical scavenging test was carried out as described by Miliauskas *et al.*[7] with a little modification. Extracts samples (300µl) at different concentrations was added to 250 µl DPPH (1 mM). After reacting for 30 minutes, the absorbance was read at 517 nm. DPPH radical scavenging was calculated using the following equation: DPPH radical scavenging (%) = [(A1 - A2) / A1] x 100 where A1 is the absorbance of the control (containing all the reagents except sample extract), and A2 is the absorbance of reagen and the sample extract.[6]

Antibacterial bioassay

Ethyl acetate extracts from endophytic fungal culture were individually tested against panels of disease-causing microorganisms including gram-positive bacteria (*B. subtillis* ATCC6633) and gram-negative bacteria (*P. aregunisa* ATCC9027 and *E. coli* ATCC8939). The inhibitory effect of extracts obtained from endophytic fungal culture on bacteria was tested by paper disc method. Disc paper (10 mm in diameter) was dipped in a solution of endophytic microbial culture extract with a concentration of 10 mg/mL (dry residue /volume of methanol), then dried and placed on NA medium for bacteria to be tested. The amount of inhibitory activity of endophytic fungi culture extracts against pathogenic microbes was assessed by measuring the diameter (in mm) of the zone of inhibition relative to positive and negative controls. Chloramphenicol were used as a positive control in treatment with pathogenic bacteria, while for negative controls methanol was used. Four disc paper replications were used on each petri disk. Petri dishes that had been inoculated with fungi and bacteria were incubated at 27 °C for 24 hours for clinical bacterial strains, antimicrobial activity was evaluated by measuring the clear zone of the test organism. Each test in this experiment was repeated three times.[8]

RESULTS AND DISCUSSION

Fermentation and extraction of endophytic fungi

In previous study, we successfully isolated 34 endophytic fungi from gall rust sengon. The best three isolate from 34 isolates obtained from gall rust sengon were identification as *Fusarium* sp A, *Fusarium* sp A and *Fusarium* sp C.

Identificatioan was carried out molecularly. The large number of isolates of endophytic fungi that can be isolated from gall rust sengon reinforce the results of Strobel and Deasy studies suggesting that all plants found are harbors of some endophytic fungi.[2] All endophytic fungi have many different functions for plants as a consequence of mutualistic symbiosis between endophytic fungi and plants. The abundance of the composition of the endophytic fungi community was strongly influenced by environmental factors (temperature and humidity), chemical variation, anatomy and maturity of the colonized host organ.[9]

How the endophytic fungus could enter the host plant without causing the disease symptoms, could be explained by Sieber[10], who explained that the initial step of endophytic fungi went into the host plant was through recognition, germination and penetration. A similar process was also experienced by infections of plant pathogenic fungi. Along the path of the process, endophytic fungi must overcome the defense mechanism of the plant. Spores of fungi often recognized host plants through molecules such as lectins. After germination, the fungus would penetrate into the plant tissue by softening the cuticle and epidermal cell wall or damaging the cuticle with mechanical strength. Once the fungus could penetrate into the plant tissue, and it would change to a latent state, the host's defense mechanisms were no longer activated. This phenomenon was explained by the Gene-for-Gene (GFG) model, in which the avirulence genes (AVR) of the endophytes were encoded into an elicitor and recognized by the product of the resistance gene (R) of the host plant as well as the hypersensitive reaction of the host plant and then the serenity the next occurs through the signal transduction path. Instead, in pathogenic fungi did not contain the AVR gene, thus product R was not produced and symptoms of the disease would develop.[10]

The interaction of endophytic fungi with host plants resulted in a compromise between mutualism and antagonism to create a harmonious symbiotic system. Plants could limit the growth of endophytes, and thus endophytes could use various mechanisms to survive. Endophytes not only described some plant metabolites with exoenzymes to take important nutrients and energy to survive, but also produced beneficial compounds and /or support or promote the growth of host plants to achieve a balanced environment.[1]

The isolated of endophytic fungi were also grown on PDB medium (pH 5.5), temperature 28 ± 2 °C, shaking condition for 21 days to determine mycelium growth. Dry weight of mycelium was used as a parameter of endophytic fungal growth. The growth of the mycelium of endophytic fungi during 21 days fermentation could be seen in Figure 1. This data showed that the endophytic fungi isolated from gall rust can grow well in the PDB medium, so it was hoped that they also produced secondary metabolite well.

The growth curve graph shows that the initial stage of growth is still at an adaptation level so that exponential growth starts on day 3 and reaches a maximum at H6. Static conditions were found in H6 - H12 and began to decrease in H15 to 21. Growth curves are measured by

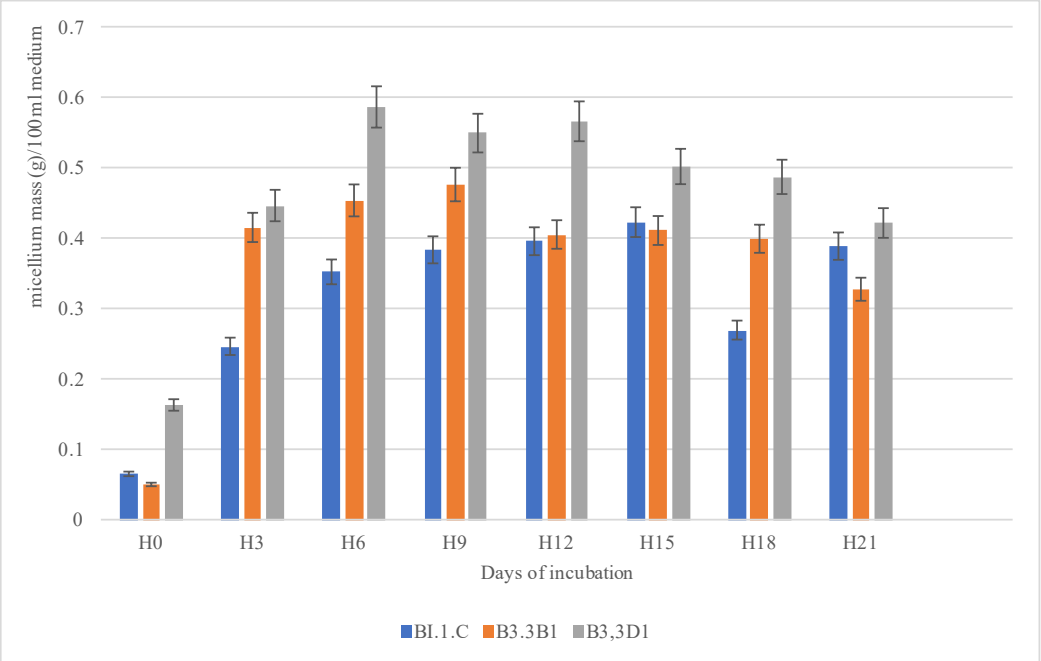

Figure 1: The growth curve of endophytic fungi from gall rust *F. moluccana* (B11C: *Fusarium* sp A, B33B1: *Fusarium* sp B, B33D1: *Fusarium* sp C).

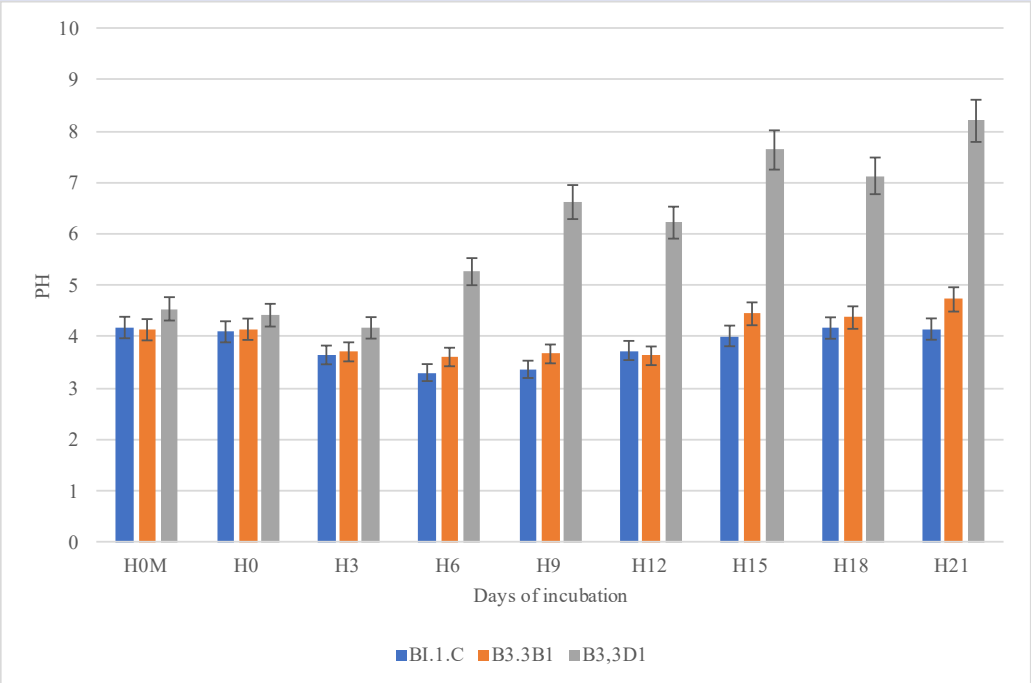

Figure 2: The change of pH as long as growth of fungi (B11C: *Fusarium* sp A, B33B1: *Fusarium* sp B, B33D1: *Fusarium* sp C).

measuring changes in mycelium weight during fermentation. At the beginning of growth the weight of mycelium is very low. When H3 starts to increase in mycelium weight and reaches its peak at H6, it stagnates to completion.

On day 3 it was seen that the pH began to decrease, this was allegedly due to the production of organic acids which could reduce the pH value. However, the H15 begins to increase in pH from acid to neutral. This phenomenon occurs in the 3 isolates used.

Based on the graph in Figure 3, *Fusarium* sp A isolate produced the highest secondary metabolite on day 15, whereas *Fusarium* sp C isolate the highest production was in H12 while B33b isolate produced the lowest secondary metabolite compared to the two other isolates. Calvo *et al*.[11] stated that environmental factors that affect secondary metabolite production such as aflatoxin production in *aspergillus* fungi are pH, carbon and nitrogen source. While Agusta[12], states that suitable environmental conditions are needed for the occurrence of the sporulation process and also a determinant of the formation

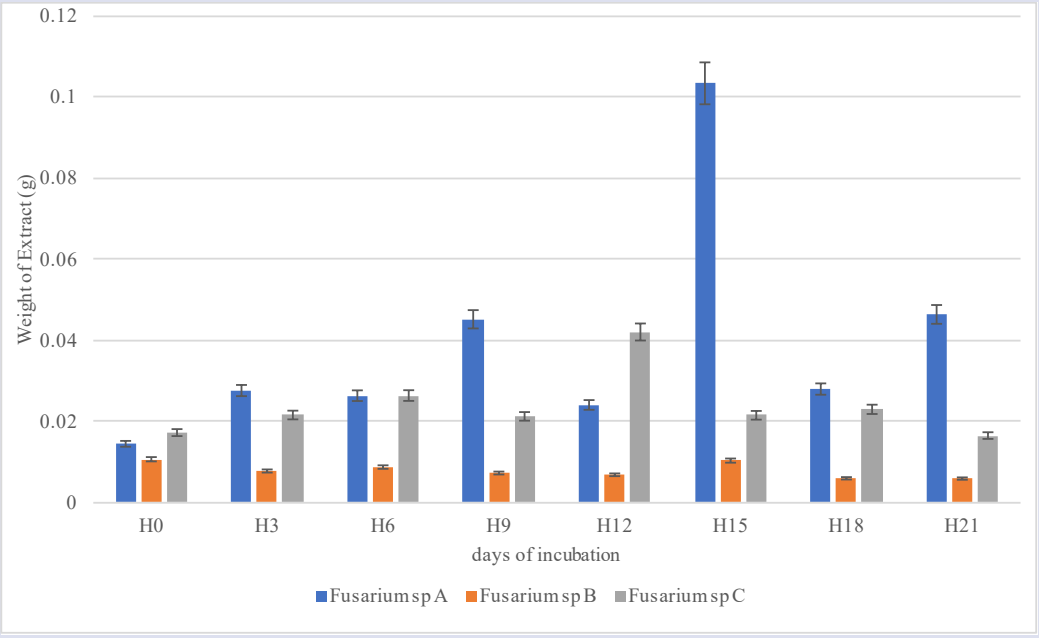

Figure 3: The curve of extraction of seconndary metabolite from endophytic fungi of gall rust *F. moluccana.*

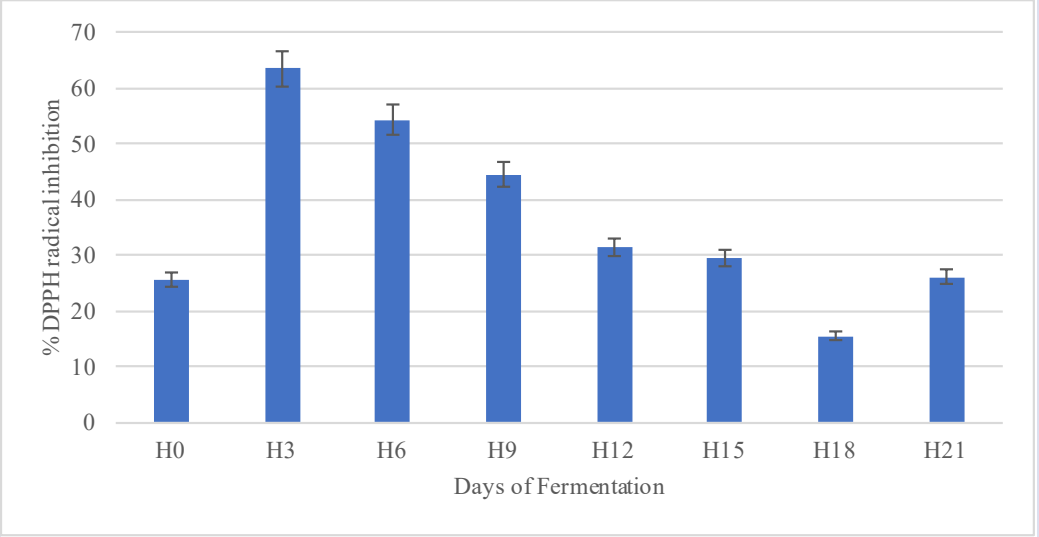

Figure 4: Graph of antioxidant activity (DPPH radical inhibition) by endophytic fungal culture extracts.

of secondary metabolites. Factors such as differences in carbon and nitrogen sources. The pH, temperature, and concentration of salt may affect the secretion of metabolite compounds.

To determine the production of antioxidant compounds during the growth period of the endophytic fungus, a dry weight measurement of extracts from ethyl acetate solvents was used. This solvent was choosed based on previous research, that showed, among 3 solvent (methanol, ethyl acetate and n hexane) that used to extrac fungal culture, ethyl acetate gave the best result.[1] Ethyl acetate solvent was used as it had medium polarity so that it could dissolve polar and non polar active compounds.

Antibacterial bioassay

The methanol solvent was a polar solvent which could dissolve almost all organic compounds, even polar. semi polar. and non polar. Ethyl acetate solvents were commonly used in extracting endophytic fungal cultures.[13] It was semi polar solvent, so it could extract the components contained in the fungal culture.

Table 1 shows that the antibacterial activity of the 3 endophytic fungi isolates against the 3 test bacteria was not the same. The highest inhibitory activity of *B. subtillis* ATCC6633 bacteria by B33d2 *Fusarium* sp C isolates was highest in H15, although H3 also showed a fairly high bacterial inhibitory activity. Antibacterial activity of ethyl acetate extract from B33d2 *Fusarium* sp C.

All the endophytic fungi of the gall rust sengon were fermented at PDB medium and the crude, EtOH extract were tested for the antimicrobial activity. Antimicrobial activity of the EtOH extract of the fungal fermentation broth at a concentration of 10 mg/mL was conducted by way of agar diffusion method against a panel of target pathogenic microorganisms, including Gram-positive (*B. subtilis*

Table 1: Inhibitory activity of 3 bacteria used (*B. subtilis* ATCC6633, *P. Aeruginosa* ATCC9027, and *E. coli* ATCC8939).

Days	B. subtilis ATCC6633 (mm)			P. aeruginosa ATCC9027 (mm)			E. coli ATCC8939 (mm)		
	Fusariun sp A	Fusarium sp B	Fusarium sp C	Fusarium sp A	Fusarium sp B	Fusarium sp C	Fusarium sp A	Fusarium sp B	Fusarium sp C
H0	0	0	0	0	0	0	0	0	0
H3	10	5	**27**	10	10	15	27.5	10	0
H6	10	6.7	27	10	20	18.3	30	15	20
H9	10	15	27	10	10	**26.7**	30	15	20
H12	10	12.5	27	10	10	18.3	25	12.5	**30**
H15	10	15	30	10	10	15	17.5	10	30
H18	15	13	30	10	10	23.3	15	10	30
H21	15	10	30	10	10	20	15	10	30
Chloram	30	30	30	30	30	30	30	30	30
Methanol	0	0	0	0	0	0	0	0	0

ATCC6633), Gram-negative bacteria (*P. aeruginosa* ATCC9027 and *E. coli* ATCC8939). Methanol was used as negative control, while chlorampenicol (100 lg/disk) as positive control, was used as standard antibacterial agents, respectively. Inhibition zones in diameter were measured to assess antimicrobial activity. Each inhibitory experiment was replicated three times**.**

Endophytic fungi living asymptomatically in plant tissues have also been recognized as a repository of novel secondary metabolites for potential therapeutic use. In this study we have demonstrated that crude extracts from the culture broth of endophytic fungi grown on PDB medium displayed considerable antimicrobial activity against a panel of microbe tested. Ethyl acetate extract from endophytic fungi culture tested on *B. subtilis* ATCC6633, *P. aeruginosa* ATCC9027 *and E. coli* ATCC8939 bacteria showed the highest anti-bacterial inhibition compared to methanol extract and hexane extract.[1]

Antioxidant activity

The highest DPPH inhibitory activity was found in H3 fermentation. The longer the fermentation time the antioxidant activity will decrease. The method commonly used to test antioxidant activity is by using a stable free radical diphenilpycrylhydrazil (DPPH). DPPH method is chosen because it is easy, fast, sensitive and requires only a small sample extract. DPPH compounds are free radicals that are stable and active by delocalizing free electrons in a molecule so that the molecule is not reactive as other free radicals. This delocalisation process is indicated by the presence of concentrated violet which can be characterized in absorbance bands in ethanol solvents at a wavelength of 517 nm.[14] In this method, DPPH solution which acts as a free radical will react with antioxidant compounds so that DPPH will turn into diphenilpycrilhydrazine which is non-radical. The parameters for interpreting the test results from the DPPH method are generally made in the form of Concentration 50 (IC_{50}), which is defined as the concentration of the substrate solution or the sample which will reduce DPPH activity by 50%. The greater the IC_{50} value, the smaller the value of antioxidant activity. An antioxidant compound is declared good if the IC_{50} value is getting smaller. Antioxidant compounds are said to be very strong if they have IC_{50} values of less than 0.05 mg/mL, strong for IC_{50} between 0.05-0.10 mg/mL, moderate for IC_{50} between 0.10-0.15 mg/mL and weak if IC_{50} is worth between 0.150-0.20 mg/mL.[14]

Endophytic fungi isolated from gall rust were screening for antioxidant activity to know the potential of the fungi to produce antioxidant compound that inhibited free radicals. The methanol extract of endophytic culture of gall rust from sengon inhibited DPPH radical 45 %, meanwile ethtyl acetate extract inhibited DPPH radicals 71.5 % and n hexane extract inhibited 28%.[1] Screening endophytic fungi from Surian resulted several isolate that potential to produce antioxidant compound, like endophytic fungi from stemand from twigs and leaves of surian.[15] Methanol, ethyl acetate and n hexane extract from bark and stem of sengon, and gall rust sengon had high antioxidant activity.[16]

Secondary metabolites from endophytic fungi can play a role in plant defense, and some to have pharmaceutical potential. Antioxidant efficacy cannot be comprehensively predicted with only a single antioxidant assay[17], but the DPPH assay is one of the most commonly used methods to evaluate free radical scavenging activity. DPPH is a stable radical that produces a purple solution in methanol. The antioxidant activity in this study was measured by the discoloration to yellow as the stable molecule 2,2-diphenyl-1-hydrazine formed. Antioxidant compounds can scavenge the radicals by donating their hydrogen[18], and the hydroxyl group of the isolated compounds may play a role in the activity. In the DPPH radical scavenging assay, antioxidants donate an electron or hydrogen radical to the DPPH radical to become a stable molecule causing discoloration of the DPPH radical.[19] Endophytic fungi from *E. sylvestris*, *Pseudocercospora* sp. ESL 02 showing the highest antioxidant activity with the antioxidant compound *Terreic acid (1)* and *6-methylsalicylic acid (2)*, with terreic acid having strong antioxidant activity. The study also complements the study of the antioxidant potency of *E. sylvestris* as the host plant of the fungus.[20]

Ethyl acetate extract culture of endophytic fungi *Aspergilus. niger* and *Alternaria. alternate* isolated from different organs of *Tabebuia argentea* showed an antioxidant capacity of 4.299 - 5.276 umol/L and total phenol of 2.5-2.6 mg/100 mL cultures equivalent to the highest phenolic acid of endophytic fungal cultures.[21] Analysis of 292 endophytic fungi isolated from 29 traditional Chinese herbs showed that the antioxidant capacity of endophytic fungal cultures was significantly correlated with the total phenol.[22]

In addition to the extensive research on bioactive compounds from plants, research on the biodiversity of fungal endophytes had also received much attention. Endophytic fungi may also produce other general secondary metabolites, such as adenosine, which exhibited potential 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities, was isolated from *Penicillium sp*. YY-20, an endophytic fungus isolated from *Ginkgo biloba*.[23] In the present study, the endophytic fungi from *E. sylvestris* were isolated and evaluated for their antioxidant potency. Furthermore, secondary metabolites having antioxidant activity were isolated and identified from the fungus that exhibited the highest antioxidant potency. This study can complement the research on the antioxidant potency of *E. sylvestris* as the host plant of the endophytes.[1]

CONCLUSION

The growth phase of endophytic fungi isolated from gall rust sengon consist of adaptation, logaritmic, stationary and death phase. Production of secondary metabolite began high at day 9, reached the peak at day 12-15th. The best activity for antibacterial was reached at day

9-12. The best production secondary metabolite that has high activity was at Day 9-12.

ACKNOWLEDGMENTS

The authors would like to thank the Directorate of Research and the Community Service Directorate General of Research and Development of the Ministry of Research Technology and Higher Education Republic of Indonesia, for providing Research Grant No. 2/EI/KP.PTNBH/2019 for this research.

CONFLICTS OF INTEREST

There are no conflicts of interest.

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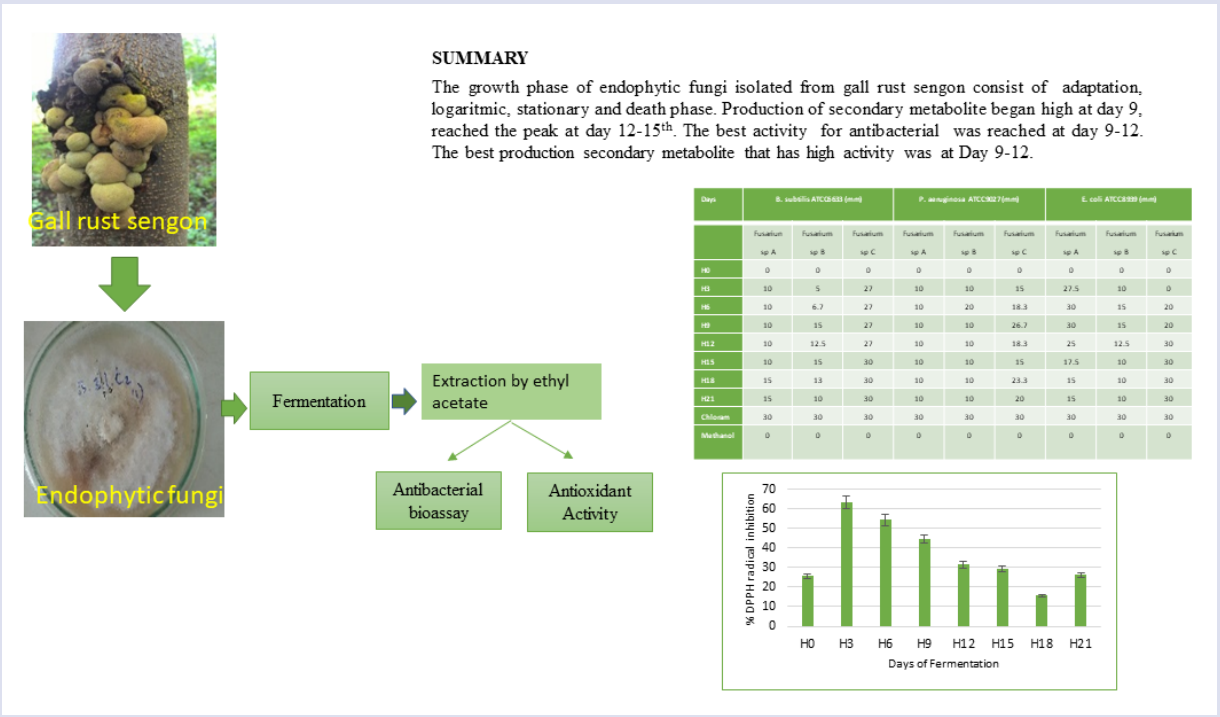
GRAPHICAL ABSTRACT



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Cite this article: Rumidatul A, Rahmawati N, Sunarya S. Production of Secondary Metabolites and its Antibacterial and Antioxidant Activity During the Growth Period of Endophytic Fungi Isolated from Gall Rust Sengon Plants. Pharmacog J. 2021;13(2): 325-31.